Infection of Guinea Pigs with Vesicular Stomatitis New Jersey Virus Transmitted by *Culicoides sonorensis* (Diptera: Ceratopogonidae)

ADALBERTO A. PÉREZ DE LEÓN,[1, 2] DONAL O'TOOLE,[3] AND WALTER J. TABACHNICK[1,4,5]

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ABSTRACT Intrathoracically inoculated *Culicoides sonorensis* Wirth & Jones were capable of transmitting vesicular stomatitis New Jersey virus (family *Rhabdoviridae*, genus *Vesiculovirus*, VSNJV) during blood feeding on the abdomen of six guinea pigs. None of the guinea pigs infected in this manner developed clinical signs of vesicular stomatitis despite seroconversion for VSNJV. Guinea pigs infected by intradermal inoculations of VSNJV in the abdomen also failed to develop clinical signs of vesicular stomatitis. Three guinea pigs given intradermal inoculations of VSNJV in the foot pad developed lesions typical of vesicular stomatitis. Transmission by the bite of *C. sonorensis* may have facilitated guinea pig infection with VSNJV because a single infected *C. sonorensis* caused seroconversion and all guinea pigs infected by insect bite seroconverted compared with 50% of the guinea pigs infected by intradermal inoculation with a higher titer VSNJV inoculum. The role of *C. sonorensis* in the transmission of VSNJV is discussed.

KEY WORDS vesicular stomatitis, *Culicoides*, vector competence

Vesicular stomatitis is a disease of livestock, wildlife, and humans caused by viruses belonging to the genus *Vesiculovirus* of the family *Rhabdoviridae.* Vesicular stomatitis virus (VSV) occurs only in the Western Hemisphere. The northern most range of vesicular stomatitis is the western United States where the serotypes Indiana (VSIV) and New Jersey (VSNJV) have caused epidemics in livestock. Vesicular stomatitis outbreaks cause economic loss associated with clinical vesicular stomatitis, and livestock and horse owners also are affected by restrictions on animal movement and trade imposed by national and international veterinary disease control agencies (Bridges et al. 1997). The World Organization for Animal Health places vesicular stomatitis on its List A because it has the potential of rapid spread across international borders with substantial impact on livestock industry economies. The clinical resemblance of vesicular stomatitis to foot-and-mouth disease in ruminants requires immediate differential diagnosis of reported cases among cloven-hoofed animals.

The mode of transmission during outbreaks remains unclear. Direct contact and insect vectors were important routes of VSNJV spread during the 1995 outbreak in the western United States (Bridges et al. 1997). Insect control was one of several recommended measures to prevent horse and livestock exposure during an outbreak in 1997 of VSIV in the western United States. VSIV and VSNJV have been isolated from several insect species during outbreaks in the western United States. Recovery of VSNJV from nonbiting flies suggested that mechanical transmission may occur (Francy et al. 1988). Isolation of VSIV from mosquitoes and VSNJV from biting midges and black flies suggested that biting flies may act as biological vectors (Kramer et al. 1990, Schmidtmann et al. 1998). The transmission of VSNJV to susceptible animals by hematophagous arthropods has been demonstrated for the black fly *Simulium vittatum* Zetterstedt (Mead et al. 1999, 2004). Nunamaker et al. (2000) showed that *Culicoides sonorensis* Wirth & Jones can be infected with VSNJV in the laboratory by using an artificial blood feeding system. Drolet et al. (2005) described VSV replication and tissue distribution in *C. sonorensis* with virus widely disseminated to many organs and tissues, suggesting that transmission was likely. Intrathoracically inoculated *C. sonorensis* transmitted VSNJV to cattle (Pérez de León and Tabachnick 2006). The current study was conducted to determine whether VSNJV infected *C. sonorensis* can indeed transmit and infect guinea pigs during blood feeding. In this study, we used intrathoracically inoculated insects to ensure *C. sonorensis* infected with VSNJV. Although we assume that intrathoracic inoculation has little to no effect on VSNJV transmission and pathogenesis, this has yet to be determined. In addition, we tested the hypothesis that insect blood feeding might facilitate VSNJV transmission to animals compared

[1] Arthropod-borne Animal Diseases Research Laboratory, USDA–ARS, P.O. Box 3965, Laramie, WY 82071–3965.
[2] SCYNEXIS, P.O. Box 12878, Research Triangle Park, NC 27709–2878.
[3] Department of Veterinary Science and Wyoming State Veterinary Laboratory, University of Wyoming, Laramie, WY 82071.
[4] Florida Medical Entomology Laboratory, Department of Entomology and Nematology, University of Florida, IFAS, 200 9th St. SE, Vero Beach, FL 32962.
[5] Corresponding author, e-mail: wjt@mail.ifas.ufl.edu.

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with inoculation via syringe as has been observed for other arthropod-borne pathogens.

Materials and Methods

Guinea Pigs. Hartley male guinea pigs, *Cavia porcellus*, weighing 500–1,000 g were purchased from the Wyoming State Veterinary Laboratory (Laramie, WY), and Hilltop Lab Animals, Inc. (Scottdale, PA). The guinea pigs were never previously exposed to biting insects. Guinea pigs were kept individually in separate cages with HEPA filter covers and were handled under agricultural biosafety level (BL)-3 containment following protocols approved by the Animal Care and Use Committee of the University of Wyoming.

Insect feedings, intradermal injections, and blood collection by cardiac puncture were conducted in guinea pigs under anesthesia achieved by intraperitoneal administration of a ketamine/xylazine mixture (O'Toole et al. 2003). Guinea pigs were euthanized by exposure to CO_2 in a closed container. Unless otherwise noted, the guinea pigs were euthanized 8 d after initial exposure to inoculated or uninoculated insects and virus injection in the abdomen as described below.

Virus. The strain 8/82-CO-C of VSNJV isolated from *C. sonorensis* during the 1982–1983 epizootic in Colorado (Walton et al. 1987) was used to inoculate the insects and guinea pigs. Stock virus was passaged once in African green monkey kidney (Vero) cells and diluted with Eagle's minimal essential medium (10% fetal bovine serum, 200 U/ml penicillin, 200 μg/ml streptomycin, 100 μg/ml gentamycin, 100 μg/ml neomycin, and 5 μg/ml amphotericin B) as needed to produce the desired titer in the inocula. Virus suspensions containing $\approx 10^{8.5}$ or $10^{4.5}$ median tissue culture infective doses ($TCID_{50}$)/ml were used as indicated.

Insects. Female biting midges, *C. sonorensis* (AK colony), used in these studies were reared to the adult stage and maintained using standard procedures (Hunt 1994). The AK colony was shown to be free of VSNJV (O'Toole et al. 2003). Two- to 3-d-old biting midges were infected by intrathoracic inoculation with VSNJV under BL-3 conditions. Inoculated biting midges were held in an incubator for a minimum of 10 d at 20°C, 70% RH, and a photoperiod of 13:11 (L:D) h with free access to a 10% sucrose solution. Two-day-old uninoculated and inoculated adults were allowed to blood feed on guinea pigs after being placed in a cylindrical plastic cage with an inner diameter of 4.4 cm and a fine mesh at one of its ends. Insects blood-fed through the fine mesh adjoined to the guinea pig's clipped abdominal skin for 30 min. Thereafter, biting midges were anesthetized with CO_2 and placed on a chill table for inspection under a dissecting microscope to determine the proportion that took a bloodmeal. Inoculated insects were stored at −70°C after blood feeding. Uninoculated insects that blood fed on guinea pigs previously exposed to virus were also frozen at −70°C after incubation for 12 d as described above.

Virus Transmission, Acquisition, and Injection. Transmission was attempted by exposing six guinea pigs to inoculated biting midges. Insects (1,571) were inoculated with a suspension of VSNJV that contained $10^{8.5}$ $TCID_{50}$/ml. The 910 biting midges surviving the incubation period were sorted into six groups of 125–180 midges. Each group was allowed to feed on a single guinea pig (no. 1–6) on day 0. Guinea pigs 1 and 2 were exposed to additional inoculated midges on day 1 to increase the numbers allowed to feed on these animals. The abdomen was selected as the feeding site for convenience and to reflect a likely site of feeding in nature.

The ability of uninfected *C. sonorensis* to become infected through feeding on guinea pigs previously exposed to VSNJV through insect bite was tested using uninoculated insects. Eight days after exposure to inoculated insects, guinea pigs 4, 5, and 6 were used to blood feed uninoculated, uninfected insects. Each guinea pig was exposed to 442–583 uninoculated insects by using the same abdominal area where the inoculated insects fed previously.

We had determined previously (unpublished data) that the extract of one whole biting midge contained $\approx 10^{2.5}$ $TCID_{50}$. Thus, each of six guinea pigs (7–12) was exposed to virus by intradermal injection in the abdominal skin with 1 ml of a VSNJV suspension containing $10^{4.5}$ $TCID_{50}$. This dose was chosen to mimic a maximum viral load likely transmitted by 100 insects, and the site of feeding on the abdomen to reflect a likely natural route of virus deposition during insect feeding. Five 200-μl aliquots were injected in five adjacent sites to deliver the total dose. Eight days after virus exposure, guinea pigs 10, 11, and 12 were used to feed uninoculated insects at the injection site area to determine virus acquisition by uninfected insects. Each guinea pig was exposed to 355–370 uninoculated uninfected insects that fed at the area where injections were applied previously.

Three guinea pigs (13–15) were treated as positive controls by intradermal inoculation in the foot pads with a total dose of 1 ml of VSNJV containing $10^{8.5}$ $TCID_{50}$. Each guinea pig received three and two 200-μl injections in the left and right foot pads, respectively. The guinea pigs were euthanized 6 d after inoculation.

Three guinea pigs (16–18) served as negative controls. Each guinea pig received five intradermal injections of 200 μl of tissue culture medium in the abdomen. These guinea pigs were euthanized 14 d after inoculation. Another guinea pig (19) was exposed to uninoculated uninfected biting midges to assess the effect of insect feeding at feeding sites.

Assessment of VSNJV Infection. Virus, immunological, and histological assays were performed to monitor the pathobiology and development of infection in guinea pigs and insects. After euthanasia, guinea pigs were necropsied and a comprehensive range of tissues was collected from major organ systems. Samples included skin from multiple sites and oral mucosa. Pieces of skin from the insect-feeding site in the right ventral aspect of the abdomen were flattened on paper

cards and fixed in 10% neutral buffered formalin. Fixed tissues were embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin for histopathological analysis. Additionally, samples of heart, lung, spleen, liver, and kidney were obtained aseptically to assess the extent of infection. Tissues were homogenized in 4 ml of Eagle's minimal essential medium, and 1 ml of supernatant obtained after centrifugation at 2500 $\times$ *g* was inoculated into monolayers of Vero cells. Duplicates of the tissue samples were passaged three times before virus assay.

Pieces of skin from feeding or inoculation sites in the abdomen and tarsal pads were collected for immunohistochemistry and fixed in 10% neutral-buffered formalin for 10 h or less, dehydrated using an automated tissue processor, and processed into paraffin wax blocks. Viral antigen was detected using a commercial avidin-biotin kit (Vector ABC Elite, Vector Laboratories, Burlingame, CA) and chromagen 3-amino-9-ethocarbozole. Briefly, sections of skin were cut 5 μm thick and placed on positively charged slides. Sections were treated with hydrogen peroxide, flooded with normal equine serum and incubated in a 1:2000 dilution of anti-VSNJV hyperimmune mouse ascites fluid. Washed sections were incubated with a biotinylated equine anti-mouse antibody solution followed by the avidin-biotin complex and chromagen. Sections were counterstained with Harris hematoxylin and coverslipped in aqueous mounting medium.

Blood-fed inoculated insects were assayed for virus infection. Uninoculated insects surviving the incubation period after blood feeding on guinea pigs exposed to VSNJV also were tested for virus infection. Frozen insects were thawed and ground individually in culture medium. Insect homogenates were clarified by centrifugation at 12,000 $\times$ *g*, and a portion was diluted 1:10 with Eagle's minimal essential medium to inoculate Vero cells in microplates. Cell monolayers were inspected for cytopathogenic effects (CPE) at 3 d postinoculation to assess infection.

Plasma or serum samples were obtained from blood collected by cardiac puncture before experimentation and immediately before euthanasia. Plasma and serum samples were assayed for the presence of VSNJV antibodies using a competitive-enzyme-linked immunosorbent assay (c-ELISA) (Katz et al. 1995). The VSNJV c-ELISA measures the ability of a sample to reduce the binding of mouse antibodies to a recombinant VSNJV antigen. Samples with a >50% reduction value for VSNJV antibodies and serum neutralization (SN) tests with a titer of 16 were considered positive (Katz et al. 1995). The guinea pigs were negative for VSNJV antibodies before the experiments started. All the negative control guinea pigs, and the guinea pigs used to assess the effect of feeding by uninoculated insects did not develop VSNJV antibodies.

Results

Insect Blood Feeding and Infection Rates. The number of inoculated biting midges that fed on guinea pigs is shown in Table 1. Blood feeding (bf) rates for inoculated insects ranged from 36 to 80%. Virus infection was determined in inoculated insects that took a bloodmeal (Table 2). Infection rates were significantly different between the six guinea pigs, ranging from 1 to 55% ($\chi^2 = 59.57$, df = 4, $P < 0.01$). The average feeding rate for uninoculated uninfected insects taking a bloodmeal from guinea pigs ($n = 3$) previously exposed to inoculated insects or after abdominal virus injection was 69 (range 61–74%) and 73% (range 62–82%), respectively. The blood-fed uninoculated midges were negative for virus infection (Table 2).

Transmission of VSNJV by *C. sonorensis*. All the guinea pigs exposed to VSNJV via insect bite developed a positive virus-specific antibody response (Table 1). Concordance of a positive signal for SN and c-ELISA antibodies at 8 d postexposure (DPE) indicates that guinea pigs were infected with VSNJV transmitted by the biting midge, *C. sonorensis.* However, VSNJV was not isolated from tissues or blood sampled at necropsy. The weakest positive response in the c-ELISA was detected in the guinea pig where only 1% of the blood-fed inoculated biting midges were virus positive. Vesicular lesions did not develop in the skin of guinea pigs (1–6) after exposure to inoculated midges. There was no histological or immunochemical evidence of VSNJV in the skin of guinea pigs collected at necropsy.

Infection of Guinea Pigs by Injection. Bilateral vesicular lesions (1.7–2.3 by 0.7 cm) developed at 4 DPE in the three guinea pigs (13–15) inoculated with VSNJV in the plantar pads. The vesicles elicited by the 8/82-CO-C VSNJV strain were typical of vesicular stomatitis virus infection in guinea pigs injected in the foot pad. Microscopically, the lesions were characterized by moderate acute necrotizing vesiculobullous dermatitis. Intracytoplasmic viral antigen was present in ballooned keratinocytes at the roof of the vesicles. Vesicular lesions were beginning to resolve by the time of necropsy due to leakage and/or absorption of intrabullous fluid. Secondary lesions did not develop elsewhere. SN and c-ELISA reactive antibodies were detected when the guinea pigs were euthanized at 6 DPE (Table 1).

The infection of guinea pigs with an amount of VSNJV and route of inoculation mimicking transmission by 100 blood-fed inoculated insects was inconsistent (Table 1, guinea pigs 7–12). Fifty percent of the guinea pigs receiving intradermal injections of virus in the abdomen showed an antibody response which is not significantly less then the 100% positive results observed in animals exposed to inoculated insects ($P = 0.09$, Fisher exact test). Vesicular lesions also failed to develop in the abdominal skin of guinea pigs (7–12) where virus was injected.

Papular Dermatitis at Insect Feeding Sites. Guinea pigs exposed to inoculated and uninoculated biting midges developed mild acute multifocal papular (≤1–2 mm in diameter) dermatitis at insect feeding sites in the abdomen. Papules developed around 5 DPE to midges and persisted up to the time animals were euthanized. There was no ultrastructural evi-

Table 1. Humoral immune response of guinea pigs after exposure to VSNJ via insect bite and injection, and feeding by uninoculated biting midges

Guinea pig[a]	No. biting midges						Antibody response[b]		
	Transmission feeding			Acquisition feeding					
	Day	*n*	bf	Day	*n*	bf	SN	c-ELISA	Response
Group 1, inoculated midges									
1	0	147	67		0		16	55	+
	1	33	13						
2	0	110	85		0		>512	62	+
	1	31	13						
3	0	125	100		0		16	51	+
4	0	159	76	8	498	367	64	61	+
5	0	155	55	8	442	268	128	56	+
6	0	150	75	8	583	427	256	65	+
Group 2, abdominal inoculation									
7		0			0		<8	<50	−
8		0			0		16	50	+
9		0			0		64	50	+
10		0		8	370	275	8	<50	−
11		0		8	365	225	<8	<50	−
12		0		8	355	290	256	53	+
Group 3, foot pad inoculation (positive control)									
13		0			0		128	80	+
14		0			0		128	67	+
15		0			0		≥1024	55	+
Group 4, sham inoculation (negative control)									
16		0			0		<8	<50	−
17		0			0		<8	<50	−
18		0			0		<8	<50	−
Group 5, uninoculated midges									
19	0	512	418		0		<8	<50	−

[a] Guinea pigs 1–6 were exposed to inoculated biting midges during transmission feeding. Exposure during acquisition feeding was to uninoculated midges. Animals (7–9 and 13–18) were not exposed to midges and were inoculated on day 0. Guinea pig 19 was exposed to uninoculated midges to assess the effect of insect feeding.

[b] Results from serum or plasma samples from guinea pigs euthanized in each group on the indicated day after initial treatment as follows: groups 1, 2, and 5: 8; group 3: 6; and group 4: 14.

dence of rhabdoviruses in insect-induced papules. Details of the papular dermatitis induced in guinea pigs by biting midges are reported previously (O'Toole et al. 2003).

Discussion

Our experiments show that guinea pigs can be infected with VSNJV through the bite of intrathoracically inoculated *C. sonorensis.* However, guinea pigs infected with VSNJV transmitted by *C. sonorensis* did not develop clinical signs of vesicular stomatitis nor did they serve as a source of infection for uninoculated uninfected *C. sonorensis.* The failure to induce clinical disease and lesions typical of vesicular stomatitis through insect bite may be due to the small amount of virus transmitted by the insects, or the difficulty of producing cutaneous lesions in guinea pigs at sites

Table 2. Infection rates of inoculated and uninoculated biting midges that blood fed on guinea pigs

Guinea pig no.	Biting midges					
	Inoculated			Uninoculated[a]		
	Infected	Uninfected	% infected	Infected	Uninfected	% infected
1	28	52	32	NA	NA	NA
2	3	95	3	NA	NA	NA
3	1	99	1	NA	NA	NA
4	13	63	17	0	100	0
5	30	25	55	0	113	0
6	11	64	15	0	130	0
10	NA	NA	NA	0	100	0
11	NA	NA	NA	0	156	0
12	NA	NA	NA	0	170	0

NA, not applicable.

[a] Guinea pigs used to feed the uninoculated biting midges had been exposed to virus via insect bite or intradermal injection in the abdomen (see Table 1 for details). Numbers are for insects assayed for virus infection 12 d after feeding.

other then the foot pad or tongue (Skinner 1957). Transmission of VSNJV by *S. vittatum* to swine did not result in clinical vesicular stomatitis unless the feeding sites were on the snout (Mead et al. 2004). The inducement of vesicular lesions by *C. sonorensis*-transmitted VSNJV may be promoted by virus deposition in hairless skin around the mouth. Cattle infected with VSNJV through the bite of *C. sonorensis* in haired skin did not develop vesicular lesions at insect feeding sites (Pérez de León and Tabachnick 2006).

Failure to consistently infect guinea pigs with an inoculum equivalent to the amount of virus in 100 biting midges delivered by intradermal injection in the abdomen supports the suggestion that *C. sonorensis* bioactive salivary factors enhance the infectivity of VSNJV transmitted to guinea pigs (Pérez de León et al. 1997) as has been shown for mice infected by mosquito bite (Limesand et al. 2000). The amount of virus injected was likely overestimated because it has been shown that ≈11% of the total insect VSV load is found in the salivary gland – head complex of orally infected *C. sonorensis* (Drolet et al. 2005). Thus, it is likely that only a few hundred virions were transmitted to guinea pig three by the cohort of inoculated *C. sonorensis* where only one insect was shown to be virus-positive. Although probing by inoculated biting midges that otherwise failed to blood feed and produce CPE may have added to the amount of virus transmitted to guinea pig 3, we think that the amounts transmitted in this manner were insubstantial.

Limesand et al. (2003) indicated that modulation of interferon-α/β by mosquito saliva may be a critical determinant of the transmission and pathogenesis of VSNJV. Preliminary findings indicate that the salivary glands of *C. sonorensis* contain factors with immunomodulatory properties, which include the ability to inhibit macrophage nitric oxide production (Pérez de León et al. 1997). These immunomodulatory effects may be an important factor in the vector competence of *C. sonorensis* for VSNJV (Tabachnick 2000) because it likely transmits the virus extravascularly and VSV replication is inhibited by nitric oxide production (Bi and Reiss 1995). The immunomodulatory effects of *C. sonorensis* saliva on VSNJV pathogenesis require further study.

The levels of c-ELISA antibodies were similar between positive guinea pigs infected by injection or insect bite. Swine injected with an equivalent amount of virus to our inoculations developed SN antibodies between 8 and 10 DPE (Clarke et al. 1996). Perhaps the guinea pigs injected intradermally in the abdomen with negative tests at necropsy were in the process of developing SN and c-ELISA antibodies. However, guinea pigs injected in the foot pad and those exposed to intrathoracically inoculated midges developed SN and c-ELISA antibodies by 6 and 8 DPE, respectively.

Papular lesions were tested as a source of infection for uninoculated biting midges because preliminary observations (data unpublished) suggested that those lesions were involved in VSNJV infection transmitted by inoculated *C. sonorensis.* The results presented here and by O'Toole et al. (2003) demonstrated the ability of uninoculated *C. sonorensis* to induce papular dermatitis ≈5 d after feeding on guinea pigs. If viremia occurred after virus transmission by *C. sonorensis* as it has been reported elsewhere for guinea pigs injected with VSV (Arbelaez and Rocha 1983), it may have disappeared or reached insignificant levels by the time uninoculated insects took a bloodmeal. Laboratory studies showed that cofeeding of infected and noninfected black flies is another potential mechanism of VSNJV acquisition among insect vectors (Mead et al. 2000). This possibility needs to be tested in *C. sonorensis.*

Our inability to recover infectious VSNJV from tissue samples collected during necropsy was consistent with previous reports (Letchworth et al. 1996, Howerth et al. 1997). The ability to recover infectious virus depends on a variety of factors, including the inoculation site, the initial amount of virus inoculated, and the time elapsed between infection and the time of the attempted virus isolation. Virus isolation is less likely once neutralizing antibodies occur. Howerth et al. (1997) isolated infectious virus from a control swine that did not seroconvert.

Results from our laboratory studies with *C. sonorensis* support previous suggestions on the involvement of this and other biting flies in the biological transmission of VSNJV to livestock during epidemics in the western United States (Kramer et al. 1990; Schmidtmann et al. 1998). *C. sonorensis* can be experimentally infected per os (Nunamaker et al. 2000), and VSNJV transmitted by inoculated biting midges infects livestock (Pérez de León and Tabachnick 2006). Further studies are required to determine whether the course of infection with VSNJV transmitted by *C. sonorensis* is the same between inoculated and orally infected insects. However, it is still unclear how *C. sonorensis* becomes infected in nature. Nonsystemic transmission through cofeeding between infected and uninfected insects has been shown for VSV infection of black flies (Mead et al. 2004) and West Nile virus infection of mosquitoes (Higgs et al. 2005). The implication of nonsystemic transmission in the epidemiology of VSV has been discussed previously (Lord and Tabachnick 2002). This mode of transmission needs to be assessed in *C. sonorensis.* Field and laboratory data indicates that practices to protect livestock against *C. sonorensis* feeding and control measures to limit *C. sonorensis* population densities need to be an integral part of programs to control and eradicate vesicular stomatitis in the western United States.

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